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THE PHYSICAL AND CHEMICAL FATE OF FLUORIDES IN PLANTS

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THE PHYSICAL AND CHEMICAL FATE OF FLUORIDES IN PLANTS

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I INTRODUCTION

Within the last 15 years, injury to vegetation caused by fluoride emitted principally from aluminum, steel, fertilizer, ceramic, and phosphorous industries has become a serious economic problem in the United States. Burning coal (either industrial or domestic) also releases fluoride to the atmosphere. Now, the burning of rocket propellents is adding high concentrations of fluoride to the atmosphere, although these concentrations are very local and are quickly dispersed. As these industries grow, the problems relative to fluoride damage to vegetation will probably grow also.

Indirect damage to livestock due to consumption of forage with a high fluoride content has been the major economic and legal factor in the majority of fluoride problems. More information concerning the compounds involved was needed, particularly in the different types of grassy and leguminous forage. This might explain the different rates of accumulation and the different toxicity effects that have been observed in these plants.

While it seems improbable that humans eat enough salad crops to ingest a toxic amount of fluoride, some work on leafy vegetables seemed warranted, especially since there has been litigation in Oregon involving alleged injury to humans by plant-absorbed atmospheric fluoride. While considerable research had been carried out on the visible effects of fluoride on plants, it was felt that additional studies were needed to provide information on the above points.

On May 1, 1960, the National Institutes of Health granted funds to Stanford Research Institute in support of a three-year research project entitled "The Physical and Chemical Fate of Fluorides in Plants." The grant provided only a part of the total research funds needed, hence additional funds had to be raised from other sources. These were provided, in part, by the Agricultural Research Center of Stanford Research Institute the Columbia Geneva Division of United States Steel Corporation, and the Kennecott Copper Corporation.

The objectives of the study were to investigate:

1. The tissues and organs in which fluoride accumulates when absorbed from the atmosphere by leafy vegetables and forage crops.

2. The chemical and physical nature of these accumulated fluorides.

3. The effect of the absorbed fluoride on plant growth.

II SUMMARIES AND CONCLUSIONS

As a result of the research, five articles have been prepared for publication in different journals. These articles are attached and represent our reporting of the methods employed and the results obtained. The details of the work are recorded in Stanford Research Institute Notebook Nos. 5806, 5807, 5886, 6127 and 6352. The results and conclusions of the entire project can best be summarized by repeating here the summaries of the individual papers.

The Disposition of Atmospheric Fluorides by Vegetation

Leafy vegetables and forage crops were grown in greenhouses in sand culture and subjected to continuous fumigation for various lengths of time with concentrations of hydrogen fluoride varying from 0.60 $\mu g/m^3$ to 100.0 $\mu g/m^3$. The plants were divided into leaves, stems, and roots and the fluoride contents and dry weights of the various divisions determined. The data indicate that when plants absorb fluorides from the atmosphere:

1. The fluorides are almost entirely taken up by the leaf and translocated to the tip or margin of the leaf.

2. Very little, if any, fluorides are absorbed by the stems unless they contain chlorophyll (alfalfa).

3. Fluorides taken up by the leaf are not translocated downward into the stems and roots.

4. The dry weights of the tops of the plants are not affected by hydrogen fluoride fumigation concentrations as high as 100 $\mu g/m^3$ for 20 days if the leaves are not marked (spinach and endive), but they are affected if markings do develop (alfalfa, orchard grass, and romaine lettuce).

5. Under the conditions of these experiments, the dry weights of the roots appeared to be slightly reduced whether the leaves of the plants were marked or not. Because of the method of supplying nutrients, the root conditions were different from those that would be found in soil plantings; therefore, these results are not applicable to field plantings.

Some Chemical and Physical Characteristics of Fluoride Compounds After Concentration by Plants in Leaves

Plants of orchard grass, alfalfa, and romaine lettuce which had been exposed to atmospheric fluorides were dried, or frozen and then dried, then extracted with various solvents. The solvents were applied either singly, followed by water, or in successive series, ending or beginning with water. The micrograms of fluoride recovered by each solvent and remaining undissolved in the residue were determined and expressed as such or expressed as parts per million of the dry weight of the original material. The results show that:

1. Freezing the tissues increases the ease with which fluorides are extracted by the various solvents, especially water.

2. The greatest proportion of the fluorides are extracted by water whether preceded or followed by organic solvents.

3. Of the organic solvents used, the petroleum ether extracted the most fluoride from the plants, but the ratio of the amount thus extracted to the amount extracted by water was similar to the ratio obtained when pure potassium fluoride was extracted by these solvents.

4. The actual amount of fluoride unextracted by the various solvents is approximately the same for fumigated and control plants.

5. The proportion of fluorides extracted by water is much greater from the fumigated plants than from the control plants.

From all of these studies it is concluded that practically all of the fluorides absorbed as hydrogen fluoride by plants from the atmosphere is retained in living cells in a water-soluble, presumably inorganic form, although small quantities of organic fluorides appear to be present.

Some Responses of Vegetation to Atmospheric Fluorides

The results of the three experiments concerning fluoride uptake should be regarded as preliminary but suggestive of additional investigations which should be carried out to verify the results obtained as well as

expand the general knowledge of fluoride effects on vegetation. The experiments and the significant results obtained were as follows:

1. Alfalfa plants fumigated with hydrogen fluoride at night accumulated fluorides about as rapidly as plants fumigated during the day for the first three days. However, most of the fluoride accumulated at night could be washed off the plants, indicating it was adsorbed on the surface rather than taken into the plants. After three days, the plants fumigated during the day showed a much more rapid accumulation of fluoride than those fumigated at night.

2. When the roots of plants are maintained in water cultures at 30°C, the uptake of atmospheric fluoride by the tops was much less than when the roots were held at 20°C even though the air temperature surrounding the tops of the plants was identical. The results suggest that additional studies on temperature effects on fluoride uptake by vegetation would be a fruitful field of investigation.

3. Preliminary results indicate that the cell walls of plant tissues which have absorbed fluoride from the atmosphere have an increased permeability to solutes, based on electrical resistance of water extracts. Again, further study is needed to verify this point.

A Method For the Detection and Isolation of Traces of Organic Fluorine Compounds in Plants

A method for the detection and isolation of submicrogram quantities of organic fluorine compounds from plant materials in the presence of much larger amounts of inorganic fluoride is presented. The procedure consists first of a rapid screening step for use with large numbers of vegetable samples and extracts and, second, of a chromatographic step to isolate and characterize any fluoro-organics found. These methods are developed in light of specific chemical characteristics of organic fluorine compounds as a general class. A modification of Soep's quantitative sub-micro fluoride analytical method is presented as applicable to these isolation methods. Microgram quantities of organic fluorine compounds were found in the plant materials investigated but at a level too low for isolation and identification.

A Rapid Field Method for Determination of Fluoride in Plants

A colorimetric reaction, involving the cerium^{III}-alizarin complexan reagent, is described for the simple semiquantitative determination of excess amounts of fluoride. A method is outlined that may be used in the field for the determination of fluoride in plants in excess of 40 ppm.

THE DISPOSITION OF ATMOSPHERIC FLUORIDES BY VEGETATION

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Abstract--Alfalfa, orchard grass, chard, endive, spinach, and romaine lettuce were grown in sand culture and fumigated with hydrogen fluoride in the atmosphere at concentrations primarily under 1.0 μg^3. The fumigations were continuous for periods as long as four months. Under the conditions of the experiments, no significant effects of the fluorides were found on the growth of aboveground portions of the plants as long as the leaves did not develop any markings ascribable to the fluorides. The results indicated that the growth of the roots may have been suppressed slightly by the fumigations. Additional studies are needed to verify this result. Analysis of plant organs and tissues indicated that the fluorides were taken in by the leaves or chlorophyll-bearing tissue (stems of alfalfa) and translocated to the leaf extremities. No evidence of downward translocation was found.

INTRODUCTION

Emissions of fluorides to the atmosphere by various industrial operations and the direct effect of these emissions on vegetation, as well as the indirect effect on animals consuming this vegetation, have resulted in a considerable body of literature dealing with the responses of vegetation

to these fumigants, especially to hydrogen fluoride. The literature on these effects has been reviewed several times in recent years (Thomas, 1951 and 1961; Catcott, 1961; Phillips et al., 1960; and Zimmerman and Hitchcock, 1956).

In addition to the studies of visible responses of plants to fluorides investigations have also been conducted on their effect on carbohydrate content (Adams and Emerson, 1961), chlorophyll content (Newman, 1961), respiration (McNulty and Newman, 1957; Hill et al., 1959; Applegate and Adams, 1960; and Ross et al., 1962), and on photosynthesis (Thomas, 1961) of plants. Very little information has been published on the distribution of fluorides absorbed from the atmosphere into various organs and tissues of the plants. In studies carried out on corn and tomato plants, Leone et al. (1956) and Thomas (1961) indicate that the fluorides tend to accumulate in the leaves, especially the tips and margins, and very little, if any, translocation occurs from the leaves into the stems and roots.

Thus, while a great deal of work has been done on the effect of atmospheric fluorides on plants and plant metabolism, little work has been done on what happens to atmospheric fluorides when they come in contact with vegetation. How much is absorbed at the surface, and how much enters the leaf? After fluorides have entered the leaf, where do they go and in what chemical and physical form do they accumulate? The answers to these questions are important. In spite of the economic injury to a few fluoride-sensitive crops, by far the most serious effects of atmospheric fluorides

have been on animals that have consumed the plants exposed to the fluorides. Thus, it is important to know whether these fluorides remain on the outside of the plants and could therefore be washed off by a heavy rain, or whether they are absorbed into the leaf and remain there, or are translocated into the stems, roots, or fruits. It is also important to know whether any of the absorbed fluorides are converted into an organic form. It is the organic fluoride in the African plant Dichapetalum cymosum that makes it so toxic to livestock.

With these points in mind, a 3-year investigation was initiated on the physical and chemical fate of fluorides in forage and leafy vegetables fumigated with hydrogen fluoride at atmospheric concentrations of less than 1.0 $\mu g/m^3$. The study consisted of three phases:

1. The location of the fluorides on or in the plants.
2. The development of methods for demonstrating the presence of organic fluorides.
3. The chemical and physical nature of any concentration of fluorides in tissues.

This paper describes the general methods used in carrying out these investigations and the results of the first phase.

MATERIALS AND PROCEDURES

Alfalfa, orchard grass, romaine lettuce, spinach, endive, and chard were the species used in these studies. The plants were grown in sand culture supplied with a complete Hoagland nutrient solution. The seeds

were first germinated in sand and the seedlings were then transplanted to sand in two, 1-gallon glazed crocks. From 10 to 30 days were allowed for the seedlings to become established.

The fumigation treatments were carried out in two, 9- by 15-foot greenhouses, each with a total volume of 1,000 cubic feet. The greenhouses were equipped with heating and cooling equipment so that the temperature could be maintained between 70° and 80°F except during the warmest days of summer. Hygrothermographs in each house indicated actual temperatures; the same temperatures were maintained in both houses. Actually, the temperature variation was never more than 1°F. Incoming air was first filtered through two inches of ground limestone and then through three inches of activated charcoal to remove any fluorides and plant-damaging smog components. After passing the filter beds, the air was drawn past refrigeration coils for cooling. Direct refrigeration was used instead of evaporation cooling to provide the plants with growth conditions as nearly comparable to natural conditions as possible. After passing the refrigeration coils, the air stream was split--half going to one greenhouse, half to the other. Fans were directly behind the cooling coils so that the air was drawn past the filter bed and coils and forced into the greenhouses, thus giving a positive pressure inside the houses; this tended to prevent outside unfiltered air fram leaking into the houses. Because only two greenhouses were available, each served alternately as the control house from experiment to experiment so that no position effect would be confounded with treatment effect.

Except for the first experiments, all fumigations were carried out with hydrogen fluoride at less than 1.0 $\mu g/m^3$ concentration. The first experiments were carried out at higher concentrations with the deliberate intention of producing fluoride markings on at least some of the species, and used a concentration of 100 $\mu g/m^3$. The hydrogen fluoride was introduced in measured quantities into the air stream as it entered the fumigated greenhouse. Two polyethylene bottles, immersed in a constant temperature water bath, were connected in series to a metered air line by means of polyethylene tubes. The first bottle was filled with distilled water; the second, with solutions of hydrogen fluoride of known concentrations. Air was bubbled first through the distilled water to saturate it, then through the hydrogen fluoride solution to pick up the fluoride; was then led into the center of the clean air stream entering the house. By keeping the temperature of the water bath constant and by metering the rate of air flow, it was possible to maintain a fairly constant concentration of hydrogen fluoride.

The concentration of hydrogen fluoride in the greenhouse was monitored by drawing air samples of known quantity through impingers containing dilute solutions of potassium hydroxide. These air samples were then analyzed for fluorides by the method of Megregian (1951). From this analysis, the fluoride concentrations in the houses were calculated. Atmospheric samples taken at both ends of the fumigated and control greenhouses indicated a uniform distribution of fluorides.

The plants were grown for various lengths of time, depending on the requirements for each study. Once the fumigations were started, they were continuous day and night until that study was completed. (Exceptions to this were made for special studies.)

At the end of the fumigation period, the plants were harvested; the sand was washed from the roots; and on many occasions, the entire plant was washed by immersing it in a weak solution of a fluoride-free detergent and scrubbing the leaves with a camel's hair brush. The dry weights and fluoride contents of aliquots of various portions of the plants were then determined. Although the plants were divided into many segments and handled in many different ways, dry weights and total fluoride uptake could always be calculated. The dry weights were determined by drying aliquots to constant weight at 65°C. The fluoride content was determined by the Willard and Winter method (1933) as modified by Remmert et al.(1953). Thus, in addition to being able to study the distribution of the fluorides in the plants, it was also possible to study the effect, as indicated by dry weight, of the fumigation treatments on growth.

RESULTS

Plant crops were fumigated for various periods. In the first experiments, five different species (about two months olds from date of seeding) were fumigated for 20 days at a hydrogen fluoride concentration of 100 $\mu g/m^3$ in an attempt to deliberately produce fluoride-type markings. Leaves of alfalfa, orchard grass, and romaine lettuce developed typical

fluoride-type markings, and the dry weights of the fumigated plants were significantly less than those of the control plants, according to an analysis of variance. The leaves of spinach and endive showed no such markings, and the dry weights of the tops did not differ significantly from those of the control plants, even though the fumigated plants contained over 2,000 ppm, or 0.2 percent, of fluorides (see Table 1).

The plants of the alfalfa and orchard grass for which data relative to the tops are given in Table 1 were allowed to sprout from the roots and grow for two additional months under a fluoride concentration of 50 $\mu g/m^3$ to provide large quantities of high-fluoride materials for latter phases of the work. At harvest times, these plants were separated into various segments and the fluoride content of each segment determined. The results are shown in Table 2. The data show that fluoride accumulates in the blades of the leaves of alfalfa, preferentially in the petioles and stems. The alfalfa leaves with fluoride-type markings showed a somewhat higher fluoride content than the unmarked leaves on the same plants; however, these values were not significantly different from each other. The accumulation of fluoride in the tips of the orchard grass leaves is striking. It is interesting to note that the level of fluoride in the top two inches of the control plants would make them unsafe to feed to dairy cattle.

Since experiments using concentrations of 100 and 50 $\mu g/m^3$ were carried out primarily to provide materials of very high fluoride content for use in developing special methods needed in carrying out later work,

the levels of fumigation used were extremely unrealistic--being 10 to 100 times higher than the continuous concentrations found in fluoride-polluted areas. The remaining experiments were carried out at concentrations less then 1.0 $\mu g/m^3$, and in none of this remaining work did fluoride-type markings develop on any of the plants treated.

Orchard grass plants were grown in fluoride-free air for six weeks, then fumigated with hydrogen fluoride at a concentration of 0.8 $\mu g/m^3$, for five weeks. Plants one month old were also fumigated for three weeks at the same concentration. At the end of the fumigation period the leaves of the orchard grass plants were divided into three groups according to their length: over 20 inches, between 12 and 20 inches, and up to 12 inches. These lengths were then cut into the sections indicated in Table 3 and the dry weights and fluoride contents of the various sections determined. The results show that the total dry weights of the tops were not affected by the fluoride fumigation, there being no significant difference between the total weights produced by fumigated and control plants of either age group. The data do indicate that the fumigated plants produced a greater number of longer leaves and fewer shorter leaves than did the control plants. The accumulation of the fluoride in the tips of the leaves is again strikingly illustrated.

The tops of the alfalfa plants were divided into top, middle, and bottom thirds. The leaves and stems were separated and their dry weights and fluoride contents determined. The data show that fluorides tend to

accumulate in the leaves of the plants rather than in the stems, and that the fluoride content tends to be higher toward the base of the plant than toward the top. In other words, the older the plant portion (and, hence, the longer the exposure), the higher the fluoride content. The stem tissues of the plants contain chlorophyl and stomatal or lenticular openings; hence, the increased fluoride contents of the stem tissues of the fluoride-treated plants, above that of the controls, can be accounted for on the basis of direct uptake of material rather than a downward translation of material taken in by the leaves.

Although the dry weights of the leaves and stems of the control plants were slightly higher than those of the fumigated plants, these differences were not significant; in other words, the results could, by chance, have been reversed.

In another series of fumigations, plants of alfalfa and orchard grass were started in the sand cultures on November 13, 1960. On December 14, one-fourth of the plants were placed in the fumigation chamber; on January 6, another fourth; and on February 7, another fourth. The last group was maintained as a control.

The plants in the chamber were fumigated with hydrogen fluoride at an average concentration of 0.7 $\mu g/m^3$ until March 2; they were then harvested. Thus, plants of the same age were harvested which had been fumigated for 0, 23, 45, and 78 days prior to the end of the experiment. The dry weights and fluoride contents of the various portions of the plants

are shown in Table 5. Analyses of variance showed that the difference in dry weights between the roots of the orchard grass plants and the controls was significant at the five percent level. The fumigation treatments had no effect on the dry weights of the other plant parts or other species. The results show a direct relationship between fluoride content of the leaves and the length of fumigation. The fluoride contents of the roots of alfalfa seemed to be unusually high even in the control plants and to have increased significantly with time of fumigation. This suggested some slight downward movement of fluorides from the leaves.

In a further experiment, plants of alfalfa, orchard grass, romaine lettuce, spinach, and chard were grown in sand culture and fumigated with hydrogen fluoride at an average concentration of 0.60 $\mu g/m^3$. The plants were grown for eight weeks from seed and fumigated for 80 days. They were then harvested, divided into sections, and dry weights and fluoride contents were determined. The tops of the plants of all the species were washed with distilled water containing a small amount of fluoride-free detergent except that the fluoride contents of the alfalfa and orchard grass were determined on both unwashed and washed aliquots. The results of these determinations are shown in Table 6. The dry weights of the tops of any of the species did not differ significantly between the fumigation and control treatments. In some instances, the fumigated leaves and tops had a higher dry weight than the controls. The dry weights of the roots of the fumigated plants were always less than those of the

controls; however, this difference is significant at the five percent level in only one species--chard.

The data on the fluoride contents indicate that washing the plants removed from 20 to 25 percent of the fluorides from fumigated plants but had no significant effect on the content of the control plants. The leaves of chard were sectioned into portions designed to separate the periphery of the leaves from the remaining portion. The data clearly show the greatest increase in the fluoride contents of the tissues occurred in the margins of the leaves. This corresponds to the increase noted in the tips of the orchard grass leaves described earlier. The fluoride contents of the roots of the fumigated plants were somewhat higher than those of the control plants. The data on alfalfa may offer some explanation of this. When the sand was washed off the roots, many of the fine roots broke off from the heavier root parts. In harvesting the alfalfa, the small roots were deliberately separated from the main roots and added to those that were unavoidably separated during the washing. It was these small rootlets that showed the highest fluoride content in the control and fumigation treatments. Further, it was only these small rootlets that showed a significant uptake of fluorides on fumigation.

When the plants in the sand culture were watered or flushed daily, the nutrient solution was forced up through the sand, forcing out the air between the sand particles, and then allowed to drain, thus sucking in fresh air between the sand particles. In the fumigated greenhouse, this

procedure essentially amounted to fumigating the roots with fluoridated air. Thus, the fine roots as well as the tops may have been exposed to, and absorbed, atmospheric fluorides.

It is also possible that the nutrient solutions exposed to atmospheric fluorides dissolved the fluorides from the atmosphere and thus, with time, the fluoride content of the solution increased to such an extent that the plants were essentially growing in a fluoride solution. The salts used in making up the solutions also contained fluorides as impurities and this might contribute to the fluoride content of the plants. Representative samples of the nutrient solution, analyzed at the start of several experiments, were found to contain on the average about 0.2 ppm of fluoride. After one month's use in flushing the crocks in the fumigated greenhouse, the solutions contained about 0.3 ppm of fluoride. Thus, there was some increase during the treatments.

The fluoride contents of the tops for all species of the fumigated plants were three and one-half to five times as great as the roots for these plants; in the control plants, this ratio was much lower. The facts that the fluoride contents of the roots of the fumigated plants did not increase a great deal and that, in one species examined, this increase occurred in the roots most remote from the leaves indicate that fluorides taken from the atmosphere through the leaves are not translocated into the roots. The results on chard leaves indicate that these fluorides are translocated away from the stem and leaf petals into the margins of the leaves.

In another experiment, plants of alfalfa, orchard grass, chard, and romaine lettuce were grown in sand culture for 120 days from May through August and fumigated with an 0.90 $\mu g/m^3$ concentration of hydrogen fluoride. The results of the dry weight determinations and fluoride analyses on these plants are shown in Table 7. The results are essentially the same as those shown in Table 6. The dry weights of the fumigated plants did not differ significantly from those of the control plants except that they were less in the case of the roots of romaine lettuce. The fluoride contents of the roots of the fumigated plants were significantly higher than those of the control plants for only two of the four species studied. The actual increase was, at the most, 7 ppm. The fluoride contents of the leaves of the fumigated plants were always many times that of the controls. The fluoride content of the stems was somewhat higher in the fumigated plants than in the control plants, but, in actual quantities, the increase was only 8 ppm as contrasted with 170 to 218 ppm in the leaves.

DISCUSSION

The studies described here represent a group of experiments conducted to demonstrate the location of fluorides absorbed from the atmosphere into the tissues or organs of forage plants and leafy vegetables. These studies also afforded an opportunity to demonstrate the effect of atmospheric fluorides in various concentrations on the growth of the plants as measured by dry weight production.

The results indicate that there is little or no movement of absorbed atmospheric fluorides from the leaves into the stems nor from the stems into the roots. Whenever determined, the fluoride contents of the leaves were always much higher than those of the stems (see Table 4). When alfalfa plants were divided into leaves and stems from the top, middle, and bottom portion (or the youngest to the oldest portion) of the plant, the fluoride contents of leaves or stems corresponded to the age of the organs, that is, the length of time that they had been exposed to fluorides. If the fluorides had been translocated into the stems and dispersed into other portions of the plants, the fluoride content would have been more uniform throughout the plant, unless there had been a continuous flow of fluorides into the roots. The data in Table 5 show that very little, if any, accummulation of fluoride occurred in roots of fumigated plants as compared to the control plants. The data in Table 6 indicate that any increase that did occur in the rootlets appears to be a direct absorption from the culture medium and not translocation from the aboveground parts. The data on orchard grass, Table 2, and chard, Table 6, indicate that the fluorides taken up by the leaves are translocated to the tips or periphery of the leaves. These results agree with results described by Thomas (1961) and Leone et al. (1956).

Nichol et al. (1957), in describing field methods of sampling alfalfa, mention innumerable field trials which indicate that the fluoride content of the top seven inches of field alfalfa plants does not differ significantly

from the fluoride content of the entire plant. Although this result has been repeatedly obtained in field studies since then, it appears incompatible with the data given in Table 4, which show the lower portions of the plants to be much higher in fluorides than the upper portions. Two facts could account for this: In field plantings, the lower leaves tend to fall off and are not included in those portions of the plants that are cut for hay; and, in the studies reported here, the lower leaves were not shaded as in field plantings and therefore remained on the plants.

While carrying out these experiments, much effort was spent in an attempt to develop a techrique whereby tissues containing higher than normal amounts of fluorides could be determined on the basis of color development in free hand sections. Such a technique was not achieved; however, a spot-plate method was developed in which the presence of 40 ppm or more of fluorides in leaf and stem tissues was indicated. This method is described in a separate paper (Ross et al., 1963).

For all the fumigation tests conducted, the dry weights of the tops and of the total plants did not differ significantly from those of the control or nonfumigated plants except when the plants had fluoride-type markings. Although the unmarked spinach and endive plants described in Table 1 had high fluoride contents, they made as much growth as the nonfumigated plants. On two occasions, the roots of the fumigated plants made significantly less growth than the roots of the control plants, and, in all cases, the dry weights of the roots of the fumigated plants were

less than those of the control plants. This suggests that the root development may be slightly reduced even though leaves are not marked by the hydrogen fluoride fumigations. This root reduction, though significant in a few instances, was not sufficient to result in significant differences in the total growth of the plants. This, of course, is due to the fact that, in these studies, the top growth was so in excess of the root growth that variations of the top growth within the treatment were great enough to obscure any difference which might have occurred in root growth.

More work is needed to establish the effect this reduction in root growth, if it is real, would have on the productivity and longevity of various species. Because of the higher greenhouse temperatures, root growth was not favored in these experiments. No attempt should be made to apply the results reported here to field conditions. Furthermore, the method of furnishing the plants with nutrients resulted in a type of root fumigation that does not occur in the field.

SUMMARY

Leafy vegetables and forage crops were grown in greenhouses in sand culture and subjected to continuous fumigation for various lengths of time with concentrations of hydrogen fluoride varying from 0.60 $\mu g/m^3$ to 100.0 $\mu g/m^3$. The plants were divided into leaves, stems, and roots and the fluoride contents and dry weights of the various divisions determined.

The data indicate that when plants absorb fluorides from the atmosphere:

1. The fluorides are almost entirely taken up by the leaf and translocated to the tip or margin of the leaf.
2. Very little, if any, fluorides are absorbed by the stems unless they contain chlorophyll (alfalfa).
3. Fluorides taken up by the leaf are not translocated downward into the stems and roots.
4. The dry weights of the tops of the plants are not affected by hydrogen fluoride fumigation concentrations as high as 100 $\mu g/m^3$ for 20 days if the leaves are not marked (spinach and endive), but they are affected if markings do develop (alfalfa, orchard grass, and romaine lettuce).
5. Under the conditions of these experiments, the dry weights of the roots appeared to be slightly reduced whether the leaves of the plants were marked or not. Because of the method of supplying nutrients, the root conditions were different from those that would be found in soil plantings; therefore, these results are not applicable to field plantings.

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TABLE 1. DRY WEIGHTS AND FLUORIDE CONTENTS OF TOPS OF PLANTS AFTER 20-DAY HYDROGEN FLUORIDE FUMIGATION (100 $\mu g/m^3$)

Species	Dry weight per 10 plants (grams)		Fluoride content (ppm - dry weight basis)	
	Fumigated	Control	Fumigated	Control
Alfalfa	2.86	4.00*	1,943	10*
Orchard grass	1.68	2.24*	2,423	10*
Endive	0.53	0.57	2,542	51*
Romaine lettuce	0.73	1.24*	1,740	23*
Spinach	0.73	0.60	2,074	35*

*Difference between fumigated plants and controls significant at 1% level.

TABLE 2. FLUORIDE CONTENTS OF PORTIONS OF ALFALFA AND ORCHARD GRASS PLANTS AFTER 2-MONTH HYDROGEN FLUORIDE FUMIGATION (50 $\mu g/m^3$)

Species and plant part	Fluoride content (ppm)	
	Fumigated	Control
Alfalfa		
Leaves (marked)	2,580	25
Leaves (not marked)	2,202	25
Petioles	1,280	22
Stems	455	10
Orchard grass		
Leaves		
Tip 2 inches	3,571	62
2nd 2 inches	2,028	12
3rd 2 inches	1,496	9
4th 2 inches	1,004	6
5th 2 inches	521	6
6th 2 inches	334	5
7th 2 inches	172	5
8th 2 inches	100	3
Remainder	73	3

TABLE 3. DRY WEIGHTS AND FLUORIDE CONTENTS OF SEGMENTS OF ORCHARD GRASS LEAVES OF DIFFERENT LENGTHS AFTER 3-WEEK AND 5-WEEK HYDROGEN FLUORIDE FUMIGATION ($0.8\ \mu g/m^3$)

(Each value is mean of 10 plants)

Period of fumigation and leaf segment*	20" lengths		12"-20" lengths		12" or less		Total	
	Dry weight (grams)	Fluoride content (ppm)	Dry weight (grams)	Fluoride content (ppm)	Dry weight (grams)	Fluoride content (ppm)	Dry weight (grams)	Fluoride content (ppm)
5 weeks								
Fumigated plants								
No. 1	0.44	413	0.56	416	0.76	336		
No. 2	1.52	31	2.00	30	2.36	50		
No. 3	2.01	9	2.58	13	1.86	13		
No. 4	2.38	6	2.34	7	0.34	25		
No. 5	2.16	9	0.34	4				
No. 6	1.36	4						
Total	9.87		7.82		5.32		23.01	
Weighted mean		28.8		43.9		76.3		45.0
Control plants								
No. 1	0.30	92	0.54	80	1.20	63		
No. 2	1.16	8	1.88	12	3.60	14		
No. 3	1.60	4	2.44	6	2.60	8		
No. 4	1.82	7	2.10	6	0.34	9		
No. 5	1.56	6	0.20	10				
No. 6	0.68	9						
Total	7.12		7.16		7.74		22.02	
Weighted mean		10.0		11.9		19.5		14.4
3 weeks								
Fumigated plants								
No. 1	0.30	375	0.40	390	0.50	233		
No. 2	1.10	14	1.40	17	1.70	34		
No. 3	1.50	5	1.80	7	1.30	21		
No. 4	1.70	5	1.70	6	0.20	14		
No. 5	1.50	6	0.20	7				
No. 6	0.80	11						
Total	6.90		5.50		3.70		16.10	
Weighted mean		23.4		35.3		55.2		35.4
Control plants								
No. 1	0.20	54	0.40	36	0.90	15		
No. 2	0.80	6	1.30	3	2.60	5		
No. 3	1.40	2	1.70	3	1.90	3		
No. 4	1.30	2	1.50	3	0.30	6		
No. 5	1.10	3	0.10	10				
No. 6	0.50	3						
Total	5.30		5.00		5.70		16.0	
Weighted mean		4.8		5.8		5.9		5.5

*Leaf segments are numbered as follows: No. 1, top 2 inches; No. 2, second 2 inches; No. 3, third 2 inches; No. 4, fourth 2 inches; No. 5, fifth 2 inches; No. 6, sixth 2 inches.

TABLE 4. DRY WEIGHTS AND FLUORIDE CONTENTS OF ALFALFA PLANTS AFTER 5-WEEK HYDROGEN FLUORIDE FUMIGATION ($0.8\ \mu g/m^3$)

(Each value is mean of 10 replications)

Alfalfa plant part	Dry weight of 10 plants* (grams)		Fluoride content** (ppm)	
	Fumigated	Control	Fumigated	Control
Leaves				
Top	2.5	2.9	51	2
Middle	1.4	1.7	121	5
Bottom	0.9	0.8	324	23
Miscellaneous***	2.5	2.4	244	9
Total	7.3	7.8	164	7
Stems				
Top	1.7	2.3	22	2
Middle	2.3	2.6	39	3
Bottom	2.4	2.6	96	6
Miscellaneous	2.6	1.9	178	8
Total	9.0	9.4	90	5
Total leaves and stems	16.3	17.2	124	6

* Difference between fumigated plants and controls not significant.

** Difference between fumigated plants and controls significant to 1% level.

*** Small shoots emerging from the crown of the plants.

TABLE 5. DRY WEIGHTS AND FLUORIDE CONTENTS OF ORCHARD GRASS AND ALFALFA PLANTS OF THE SAME AGE AFTER DIFFERENT PERIODS OF HYDROGEN FLUORIDE FUMIGATION ($0.7\ \mu g/m^3$)

(Each value is mean of 5 replications)

Species and days of fumigation	Dry weight of 10 plants (grams)				Fluoride content (ppm)		
	Leaves	Stems	Roots	Total	Leaves	Stems	Roots
Orchard grass							
0	16.3		5.5*	21.8	5.2**		3.0
23	16.7		4.7	21.4	12.0		3.2
45	16.8		4.1	20.9	32.0		3.3
78	15.5		3.7	19.2	41.0		5.1
Alfalfa							
0	12.2	7.4	8.5	28.1	14.0**	4.0	11.0**
23	11.8	7.9	8.6	28.3	20.0	5.0	14.0
45	11.1	8.0	8.0	27.1	80.0	9.0	14.0
78	13.2	7.4	7.5	28.1	102.0	13.0	20.0

* Difference between fumigated and controls significant at 1% level.

** Difference between fumigated and controls significant at 5% level.

TABLE 6. DRY WEIGHTS AND FLUORIDE CONTENTS OF PARTS OF VARIOUS PLANTS AFTER 80-DAY HYDROGEN FLUORIDE FUMIGATION (0.6 $\mu g/m^3$)

Species and plant part	Dry weight of 10 plants (grams)		Fluoride content (ppm)			
			Fumigated		Control	
	Fumigated	Control	Unwashed	Washed	Unwashed	Washed
Alfalfa						
Tops	14.4	13.0	75	60	10	7**
Roots						
Bark	1.73	2.20		8		6
Xylem	1.40	1.55		2		2
Rootlets	1.23	1.50		41		24**
Total	18.80	18.55				
Orchard Grass						
Leaves	13.3	13.4	40	30	6	6**
Roots	4.32	4.81		8		3
Total	17.62	18.21				
Chard						
Leaves						
Tips	1.70	1.63		42		13**
Sides	3.37	3.82		36		10**
Center	3.87	4.10		10		7
Midrib	5.17	4.90		7		2
Petiole	14.4	14.9		2		2
Roots	5.20	5.80*		14		12
Total	33.95	35.15				
Romaine lettuce						
Tops	23.80	22.08		62		26**
Roots	3.00	3.84		12		8
Total	26.82	25.92				
Spinach						
Tops	5.97	5.56		52		21**
Roots	1.40	1.70		14		10
Total	7.37	7.26				

*Difference between controls and fumigated plants significant at 5% level.
**Difference between controls and fumigated plants significant at 1% level.

TABLE 7. DRY WEIGHTS AND FLUORIDE CONTENTS OF PARTS OF VARIOUS SPECIES AFTER 120-DAY HYDROGEN FLUORIDE FUMIGATION ($0.90\ \mu g/m^3$)

Species and plant part	Dry weight of 10 plants (grams)		Fluoride content (ppm)	
	Fumigated	Control	Fumigated	Control
Alfalfa				
Tops	13.20	13.27	178.0	6.2*
Roots	3.04	3.22	13.0	8.0**
Total	16.24	16.49		
Orchard grass				
Leaves	15.22	15.27	229.0	11.0*
Roots	3.09	3.17	28.0	30.0
Total	18.31	18.44		
Chard				
Leaves	13.92	16.32	215.0	11.2*
Petioles	6.48	5.76	5.5	3.7
Roots	9.00	10.26	22.0	23.0
Total	29.40	32.34		
Romaine lettuce***				
Leaves	11.08	9.44	195.0	18.0*
Stems	13.20	15.31	10.0	2.0**
Roots	2.03	2.97**	24.0	17.0**
Total	26.31	27.72		

*Difference between controls and fumigated plants significant at 1% level.
**Difference between controls and fumigated plants significant at 5% level.
***Because of the hot weather, the romaine lettuce bolted to seed; hence, the large amount of stem tissue.

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SOME CHEMICAL AND PHYSICAL CHARACTERISTICS OF FLUORIDE COMPOUNDS AFTER CONCENTRATION BY PLANTS IN LEAVES[1,2]

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A series of studies undertaken to determine the chemical and physical fate of fluorides in plants involved locating the organs and, if possible, the tissues in which fluorides taken up by vegetation ultimately accumulate, and determining the nature of the accumulated compounds. Results described in another paper (1) indicated that fluorides are primarily absorbed from the atmosphere by the leaves and are then translocated to the tips and margins of the leaves. The purpose of this paper is to present the results of studies carried out to determine something of the nature of these accumulated fluoride compounds only.

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There is very little information available as to the nature of these compounds. It has been shown that the poisonous factor of the African plant gifblaar is a fluoro-acetate (6). Attempts have been made to associate toxicity of fluorides in different species with calcium or ash content on the theory that the insoluble calcium fluoride formed might convert the fluoride in the fumigant to an inactive fluoride (4), but this association has not been definitely established. It was postulated that the soft suture condition of peaches ascribed to fluorides in the atmosphere was essentially a calcium deficiency resulting from the precipitating out of calcium fluoride in the fruit (2).

MATERIALS AND METHODS

The plant material used in these studies was obtained by fumigating plants with known concentrations of hydrogen fluoride as described by (1). If the material to be extracted was dried, the drying occurred at 65°C in a ventilated oven until a constant weight was reached. When the material was frozen, it was done so at -40°C in a deep freeze.

With the meagre information to go on it was decided to first extract with various solvents plant tissues known to be high in fluorides as a result of atmospheric fumigations but in which no necrosis had developed as a result of the fumigation. The fluorides that were extracted by the various solvents would indicate the solubility of the accumulated fluoride compounds, and from this something of their chemical nature might be

inferred. Another method of approach that was tried was to carry out analyses of plant tissues for various known fractions such as carbohydrates, then determine the fluoride content of the materials present in the various step of the processes.

RESULTS

Some preliminary trials were carried out using different methods of extraction. Extracting for long periods of time, 30 hours or so in a soxhlet extractor, appeared to give the most consistent results. This technique was superior to mixing the materials and solvents in a Waring blender. The reproducibility of the results based on several duplicate extractions resulted in coefficients of variability of about 12%.

In the first experiment, a sample of alfalfa leaves containing 324 ppm of fluorides along with a sample of orchard grass leaves composited from several lots of fumigated plants made up to contain 590 ppm of fluorides were selected for extraction. The plant samples had been dried to constant weight and then ground with a Wiley mill through a 60-mesh screen. Approximately 10 grams of each plant sample were used for the extraction. A series of solvents with varying degrees of polarity were selected beginning with petroleum ether and ending with water (3). The extraction order was then reversed on a fresh sample to determine if the order in which the solvents were used had any effect on the extraction of the fluorides. The results of the first experiment are shown in Table I. The time of extraction was 32 hours for each solvent.

The results shown in Table I indicate that the fluorides extracted by the organic solvents represent, at the most, only about 14% of the total fluorides extracted and that the fluorides extracted by the water, whether used first or last in the series, amounted to between 50%-60% of the fluorides in orchard grass and between 65%-75% of the fluorides in alfalfa. More of the fluoride remained in the residue of orchard grass than of alfalfa. In this experiment the amount extracted and determined in the residue agreed fairly well with the theoretical amount (the product of fluoride content determined by the original analysis and the weight of material used in the extraction).

In the next series of experiments, six aliquots of dried orchard grass leaves were weighed, each containing 1,800 micrograms of fluoride according to preliminary analyses of the dried plant material. Six aliquots of alfalfa tops were also weighed, each containing 1,500 micrograms of fluoride. These aliquots were first extracted for 30 hours with an organic solvent, then for an additional 30 hours with water. The micrograms of fluoride in each solvent and in the residue were determined.

The results of these extractions, as shown in Table II, again indicate that very little of the fluoride is soluble or is extracted by the organic solvents, and that most of the extractable fluorides are water soluble. The average amount of fluoride remaining in the residue of alfalfa was less than in the residue of orchard grass. When petroleum ether was used as the organic solvent, the subsequent water extraction

dissolved more fluoride than water subsequent to any other solvent. When ethanol was used the subsequent water extraction dissolved less fluorides than any other combination. This was not related to the amount of fluoride dissolved by the organic solvent, since neither ethanol or petroleum ether dissolved more than 5% of the total amount present. This suggests that the organic solvent first used may affect the water solubility of the fluorides or condition the plant material so that extractability is altered.

Although the number of micrograms of fluoride extracted varied between organic solvents, the amount dissolved by any one was so small that to draw conclusions as to the relative solubility of the fluorides in the different solvents did not seem warranted.

The results in Tables I and II are based on extraction of plant material that had been dried immediately after harvesting. It is known that freezing tissues before drying often changes solubilities. Consequently, samples of leaves and stems of alfalfa and leaves of orchard grass that had been fumigated with hydrogen fluoride and control plants were selected at the time of harvest and placed in a deep freeze at $-40^{\circ}C$ for 24 hours. They were then removed, dried at $65^{\circ}C$ to constant weight and ground in a Wiley mill through a 60-mesh screen and extracted with the series of solvents used in the experiment described in Table I. The results of this experiment are shown in Table III.

Freezing the tissues resulted in much less of the fluoride being retained in the residue, indicating that freezing before drying affects the cellular materials in such a way that the fluorides are more readily extractible following such treatments. Regardless of where it comes in the series of extractants, water dissolves the majority of the fluorides in the fumigated plants. It is interesting to note that freezing resulted in an increase in the amount of fluorides extracted by the organic solvents if the solvents were applied first. Thus in Tables I and II, only about 5% of the total fluorides accounted for were found in the petroleum ether and chloroform fractions when applied before water. In Table III, the fumigated plants had 10% or so of their fluorides dissolved in the first organic solvent applied.

The fluorides in the control plants exhibited somewhat different solubilities than those in the fumigated plants; a much higher proportion remained undissolved in either water or the organic solvents. When the fluorides are estimated on an actual amount per unit of dry weight, the only really significant differences between the control and fumigated plants occurred in the water-soluble fluorides and in the petroleum ether-soluble fraction when petroleum ether was used first in a series of solvents. When petroleum ether is the last solvent used in the series, the amount of fluorides it dissolves was the same for the control and fumigated plants. These results suggest that practically all of the increase in fluorides in plants exposed to atmospheric fluorides occurs

as a result of the buildup of water soluble fluorides. This is emphasized by the fact that, when the tissues are extracted first with water, the fluorides remaining in the residues do not differ significantly--the range being from 1.3 to 3.1 ppm, an indistinguishable difference with any methods of analyses that are now available. The fact that petroleum ether extracted some fluorides led to the speculation that some organic fluorides might accumulate in this solvent. To obtain further information on this, one gram of potassium fluoride (an inorganic salt and highly water soluble) was extracted in a soxhlet for 32 hours with a mixture of 50% petroleum ether and 50% chloroform, then for the same length of time with water. The fluoride contents of the two extracts were determined. A total amount of 0.26 grams of fluorine was recovered, which agrees reasonably well with the theoretical amount of 0.33 grams of fluorine in 1 gram of potassium fluoride. However, of the amount recovered, about 11% was recovered in the petroleum ether extract. This agrees very closely with percentage of the total fluorides found in the petroleum ether extracts when that solvent was used first in the series of extractants.

In the series of solvents used in obtaining the data in Table III, very little fluoride was obtained in the acetone fraction. There have been verbal reports that fluorides are often dissolved by acetone from green plants. In these experiments, acetone was always the mid-use solvent of the five solvents used. It might have been that the acetone-soluble fluorides would also have been soluble in the solvents which

preceded the acetone. In another experiment, leaves of romaine lettuce, which are lush and high in chlorophyll, were fumigated with hydrogen fluoride until a fluoride content of about 100 ppm had been reached. The leaves of these and a comparable set of unfumigated plants were frozen and then dried. They were extracted with acetone and then water, or with water and then acetone, and the fluoride content of the extracts determined. Enough material was available for six replications of each extraction. The results are shown in Table IV. In this experiment, the acetone, even though applied first, dissolved no more of the fluorides in proportion to the amount dissolved by the water.

Following this series of extractions with various solvents, attempts were made to study the occurrence of fluorides in various fractions that were obtained during such analyses as for carbohydrates, celluloses, proteins, and so forth. These attempts were generally unsuccessful in that the amount of fluoride recovered in the various fractions usually totaled more than the amount originally determined to be in the sample. The excess generally amounted to at least 100 percent. This seemed to be the result of interference in the fluoride analyses by reagents used for the tissue fractionation.

DISCUSSION

The results of these extraction studies all lead to the conclusion that almost all of the fluorides taken up by plants from the atmosphere accumulate in a water-soluble, presumably inorganic, form. Regardless

of the order in which the solvents are applied to the plant tissues, the greatest proportion of the fluorides that could be accounted for always occurred in the water extract and when water was applied first, well over 90% of the fluorides were extracted from frozen tissue. However, when extracts were made of plant tissues that had not been exposed to atmospheric fluorides, a much smaller proportion appeared in the water extract and a greater proportion in the organic solvent or the residue. Although the proportion of total fluorides undissolved (in the residue) varied between fumigated and control plants, the actual amounts on a dry weight part-per-million basis, were not significantly different. This suggests that it might be possible to distinguish at least in part, between fluorides taken up from the atmosphere and those taken up from the soil by extracting the plants with water. The fluorides remaining in the residue likely came from the soil.

When the tissues were not frozen, the proportion of the total amount extracted from alfalfa was always more than from orchard grass. This may be indicative in that in vegetation analyses for fluorides it has been found that the alkali fusion step is not necessary with alfalfa but is with orchard grass analyses (5).

In all the extractions there was always some fluoride found in the organic solvent extracts. This amount, however small, raised the question as to whether any of this fluoride was organic in nature. The major portion of the time and effort of the fluoride studies went into the

development of a method for detection and analysis of organic fluorides. The methods developed have been described in another paper (7). It was possible to show that organic fluorides were present in the plants fumigated with hydrogen fluoride. The amounts were so small, however, that it could not be ascertained whether their occurrence was the result of the fumigation or developed naturally from root-absorbed fluorides.

SUMMARY AND CONCLUSIONS

Plants of orchard grass, alfalfa, and romaine lettuce which had been exposed to atmospheric fluorides were dried, or frozen and then dried, then extracted with various solvents. The solvents were applied either singly, followed by water, or in successive series, ending or beginning with water. The micrograms of fluoride recovered by each solvent and remaining undissolved in the residue were determined and expressed as such or expressed as parts per million of the dry weight of the original material. The results show that:

1. Freezing the tissues increases the ease with which fluorides are extracted by the various solvents, especially water.
2. The greatest proportion of the fluorides are extracted by water whether preceded or followed by organic solvents.

3. Of the organic solvents used, the petroleum ether extracted the most fluoride from the plants, but the ratio of the amount thus extracted to the amount extracted by water was similar to the ratio obtained when pure potassium fluoride was extracted by these solvents.

4. The actual amount of fluoride unextracted by the various solvents is approximately the same for fumigated and control plants.

5. The proportion of fluorides extracted by water is much greater from the fumigated plants than from the control plants.

From all of these studies it is concluded that practically all of the fluorides absorbed as hydrogen fluoride by plants from the atmosphere is retained in living cells in a water-soluble, presumably inorganic form, although small quantities of organic fluorides appear to be present.

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Table I

Fluoride Extracted From Dried Alfalfa & Orchard Grass Leaves by Various Solvents Expressed in Parts per Million of the Dry Weight of the Original Sample & Percentage of Total Accounted For

Solvent (in order used) plus remainder	Alfalfa		Orchard Grass	
	ppm	%	ppm	%
Petroleum ether	10	3.1	23	4.2
Chloroform	9	2.8	19	3.4
Acetone	14	4.3	6	1.1
Absolute alcohol	14	4.3	35	6.3
Water	218	65.6	285	51.5
Residue	66	20.0	186	33.6
Total	331		554	
Theoretical total	324		590	
Water	221	72.7	347	60.5
Absolute alcohol	7	2.2	24	4.2
Acetone	3	1.1	7	1.2
Chloroform	12	3.8	9	1.5
Petroleum ether	6	2.0	11	1.9
Residue	52	18.1	78	30.4
Total	301		576	
Theoretical total	324		590	

Table II

Micrograms & Percentage of Total Fluorides Removed From Alfalfa & Orchard Grass by Soxhlet Extraction by Various Organic Solvents Followed by Water

Species and organic solvent	Fluorides extracted by Organic solvent		Fluorides extracted by Water		Fluorides in residue		Total fluorides
	µg	%	µg	%	µg	%	µg
Alfalfa							
Acetone	40	3.2	980	77.5	240	19.3	1,260
Ethanol	50	3.9	640	50.4	580	45.6	1,270
Ethyl ether	50	3.6	1,040	75.0	300	21.5	1,390
Petroleum ether	30	2.1	1,180	81.5	240	16.4	1,450
Benzene	160	12.9	640	51.6	440	35.5	1,240
Chloroform	60	3.9	1,020	66.6	450	29.5	1,530
Mean	65	4.8	917	67.5	375	27.7	1,357
Orchard grass							
Acetone	40	2.5	540	34.2	1,000	63.3	1,580
Ethanol	80	4.8	510	30.4	1,090	64.8	1,680
Ethyl ether	10	0.6	800	47.4	880	52.0	1,690
Petroleum ether	50	2.8	1,400	79.6	310	17.6	1,760
Benzene	80	3.8	1,370	64.6	660	21.6	2,110
Chloroform	170	9.5	1,250	69.8	370	20.7	1,790
Mean	72	4.1	978	55.3	718	40.6	1,768

Table III

Fluoride Extracted From Frozen & Then Dried Alfalfa & Orchard Grass Plants by Various Solvents Expressed in Parts per Million of the Original Dry Weight of the Tissues & Percentage of the Total Amount Determined

Solvent (in order used) plus remainder	Alfalfa				Orchard grass	
	Stems		Leaves		Leaves	
	Fumigated	Control	Fumigated	Control	Fumigated	Control
	Parts per million					
Petroleum ether	2.8	1.1	12.1	0.9	23	1.5
Chloroform	0.9	1.0	5.4	0.8	16	1.0
Acetone	1.0	0.4	0.3	0.6	1	0.2
Ethanol	0.8	0.2	0.5	0.4	1	0.2
Water	19.8	2.0	83.0	3.3	203	5.8
Residue	0.9	0.4	3.7	2.0	4	0.6
Total	26.3	5.1	105.0	8.0	248	9.1
Water	27.4	2.1	125.8	4.8	223.0	5.8
Ethanol	0.2	1.8	0.8	0.2	0.6	1.8
Acetone	0.1	0.3	0.1	0.1	0.2	0.3
Chloroform	0.1	0.4	0.1	0.1	0.2	0.2
Petroleum ether	0.1	0.5	0.1	0.1	0.2	0.2
Residue	1.2	1.6	3.1	2.3	1.3	1.5
Total	29.1	6.6	130.0	7.5	225	9.8
Theoretical total	30.0	5.5	117.0	9.0	229	11.0
	Percentage					
Petroleum ether	10.7	22.5	11.3	11.1	9.4	15.6
Chloroform	3.4	18.6	5.1	10.1	6.6	11.4
Acetone	3.7	8.6	0.3	7.4	0.4	1.8
Ethanol	3.0	4.3	0.5	5.1	0.2	1.8
Water	75.0	40.0	79.4	41.8	82.6	62.8
Residue	3.3	7.1	3.5	24.4	1.7	6.6
Water	94.8	32.2	96.7	64.3	98.7	59.7
Ethanol	0.7	27.6	0.6	3.1	0.3	18.2
Acetone	0.3	4.6	0.1	1.1	0.1	3.4
Chloroform	0.2	6.2	0.1	1.1	0.1	1.7
Petroleum ether	0.2	7.7	0.1	1.1	0.1	1.7
Residue	4.0	24.6	2.4	29.3	0.6	15.3

Table IV

Fluorides Extracted by Acetone & Water or Water & Acetone From Romaine Lettuce Leaves Expressed As Parts per Million of the Original Dry Weight & as Percentage of the Amount Extracted

Solvent (in order of use)	Fluoride content (ppm)		Percentage	
	Fumigated	Control	Fumigated	Control
Acetone	2.4	3.4	2.3	17.4
Water	100.2	16.2	97.7	82.6
Total	102.6	19.6		
Water	116.4	14.4	98.4	93.5
Acetone	2.6	1.0	1.6	6.5
Total	118.0	15.4		

SOME RESPONSES OF VEGETATION TO ATMOSPHERIC FLUORIDES*

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Introduction

During the course of a series of investigations conducted under the general title of "The Physical and Chemical Fate of Fluorides in Plants," some miscellaneous single investigations were completed. Since they yielded useful information and suggested lines along which additional research might be pursued, three of these studies are reported here. Two deal with effects of environmental conditions on fluoride uptake from the atmosphere and the third with a possible effect of internal fluorides on cell wall permeability.

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That fluorides are readily absorbed from the atmosphere by plants has been well known for many years.[1,2,3] Some studies have been carried out on the effect of various environmental conditions on the rate of this absorption. Adams, Hendrix, and Applegate[4] report that dark fumigation was over 90% as effective as daylight fumigation in promoting foliar fluoride burn. Benedict and Breen[5] have shown that plants growing under drought conditions are not as sensitive to marking by atmospheric fluorides as plants grown with abundant moisture. This, however, does not necessarily mean that the drought-treated plants absorbed less fluoride from the atmosphere. Applegate and Adams[6] and Leone et al.[7] have studied the effect of available nutrients on the uptake of fluorides both from the nutrient solution and from the atmosphere. Spraying plant parts with lime inhibits the development of fluoride necrosis, presumably as a result of the reaction with fluorides to form CaF_2 which is insoluble and unavailable to the plant tissues.[8,9]

Fluoride-type markings usually occur on the margins or tips of leaves and resemble in a very superficial manner the type of markings produced by unusual amounts of salt accumulations in leaf tissues or that produced by rapid desiccation due to high winds in hot weather. Hence, it was postulated that fluoride accumulation in cells might effect the permeability of the cell walls of the tissue. For these reasons, some preliminary studies were undertaken on the effects of fluorides on cell wall permeability along with the studies on the effects of some environmental conditions on fluoride uptake.

Methods

The plants used in these studies were grown in temperature-controlled greenhouses equipped with limestone and carbon filters to remove any fluoride and smog from the greenhouse atmosphere. The method of fumigating, harvesting, and determining the fluoride contents of the plants have been described in a previous paper by Benedict et al.[10] Special methods were involved for each experiment and these are described as appropriate in the results section.

Results

Fluoride Uptake During Day and Night

Alfalfa plants growing in soil were fumigated for three and five 8-hour periods--8:30 a.m. to 4:30 p.m. during the day or from 8:00 p.m. to 4:00 a.m. during the night. The concentration of fluoride used was 5.0 $\mu g/m^3$. The fumigations were carried out during the late fall days when the days were short enough to ensure complete darkness during the nighttime exposures. The fumigations were all carried out at 22°C. At the close of the exposure period, the tops of the plants were harvested and divided into two aliquots. The fluoride content of the first aliquot was determined immediately. The second aliquot was carefully washed in a weak, fluoride-free detergent solution and the fluoride contents of washed plants and the wash water determined. These were then totaled, and the total compared with the results of the analyses on the first aliquot.

A porometer in connection with microscopic examinations of the leaves indicated that the stomata of the plants were closed during the night fumigations and open during the day.

The results of the fluoride determinations are shown in Table I. After 24 hours the total fluoride content of the plants fumigated at night did not differ from that of those fumigated in the daytime. However, the plants fumigated during the day had almost 2.5 times as much fluoride as those fumigated at night. After five periods of exposure, the total fluoride content of the day-fumigated plants was significantly greater than that of the night-fumigated plants. However, of the total amount found with the night fumigated plants, less than half was in the plant, the rest was removed by washing. With the day-fumigated plants, more than two-thirds of the fluoride accumulated was in the plant, and only one-third could be removed by washing.

The most striking result is that the plants fumigated at night showed no significant increase in fluoride content between the 24- and 40-hour exposures whereas the fluoride content of the plants fumigated during the day doubled as a result of the additional fumigation.

The results indicate that, during the night when the stomata are closed, the plants take in little atmospheric fluorides, but that more is adsorbed on the outside of the plant and is removable by washing.

Fluoride Uptake by Plants

The method used to determine fluoride uptake by plants consisted of growing the plants hydroponically and controlling the temperature of the nutrient solution. Three separate water baths were employed which maintained the temperatures at 20°C, 25°C, and 30°C, respectively. The plants were grown in 1-gallon polyethylene freezing containers with four plants per container. Four of these containers could be placed in each water bath and the four species grown simultaneously.

Fumigation was begun as soon as the seedlings were transplanted to the hydroponic solution and the plants were allowed to grow for about two months. The fluoride concentration in the green house where the plants were grown was maintained at 0.8 $\mu g/m^3$.

The results of these experiments are shown in Table II. The effect of the root temperature on the growth of the roots and tops is apparent. In general, the roots had the greatest dry weight at the lower temperatures, with orchard grass being the exception. As far as top growth is concerned, the higher root temperatures favored growth of the tops of orchard grass and romaine lettuce and inhibited the growth of the tops of chard and alfalfa.

The results of determining the fluoride contents of the roots and tops on a unit dry weight or ppm basis indicate that the amount found in the roots at 30°C was always more than double that found in the roots at

20°C. On the other hand, the amount found in the tops at the higher root temperature was less than that found at the lower root temperatures.

The fluoride contents of the roots were related to some extent to the nature of the roots themselves. The roots of the alfalfa are very coarse and, especially in water culture, had no fine roots but only a single tap root. The roots of the other plants, however, had many fine roots and much higher root-fluoride contents.

The fluoride contents of all the roots were high and the general inverse relationship between the fluoride contents of the roots and tops suggest that the high root temperature favors the translocation of the fluoride from the tops to the roots. This is to be expected since the tops of the plants are the portions exposed to the atmospheric fluorides. However, previous work in these laboratories[10] and elsewhere[7] has provided rather strong evidence that fluorides absorbed from the atmosphere are not translocated from the tops to the roots.

Another possible explanation for the apparent translocation was that the air bubbling through the water bath for root aeration purposes resulted in a marked increase in the fluoride content of the nutrient solution and that these dissolved fluorides entered or were adsorbed on the plant roots. However, analyses of the solution at the start of the experiments showed a range between 0.1 and 0.2 ppm of fluoride and when the solution was changed after one month the analysis was still in this range. It is possible, of course, that the plants removed fluoride as fast as it was

accumulated. Additional work must be carried out to reconcile the results obtained here with those reported elsewhere.

The only conclusion that can be drawn from these results is that the temperature at which the roots are growing may readily affect the rate of fluoride uptake by the tops of the plants.

Effect of Fluorides on Cell Wall Permeability

This series of studies was concerned with the possible effect on the permeability of the cell walls of tissues of plants exposed to atmospheric fluorides. A possible approach was to measure the rate of change in conductivity of distilled water into which sections of stems or roots of plants exposed and not exposed to atmospheric fluorides were placed. The conductivity or electrical resistance of such water would be dependent upon the amount of solutes which diffused out of the plant tissues. The more that diffused out, the greater the permeability of the cell walls and the greater the conductivity, or the less the electrical resistance of the water.

Freshly harvested alfalfa roots and chard crowns were cut into pieces to give the same weight and surface area by pressing a sharpened steel cork borer transversely into the tissues. The resulting plug was removed and cut into the same number of sections to give equal weights for each measurement. The excised root sections were immediately immersed in 100 ml of distilled conductivity water and the electrical resistance

change was recorded versus time. The flasks with the sectioned roots and water were immersed in a 25°C water bath during the measurements. It was found that the values would vary significantly unless the roots were transferred immediately to the measurement flask.

The plants used for the experiment were grown in the same nutrient solution (Knop's) as were the hydroponically-grown specimens from the previous experiment. Four replications were made for each species studied.

The resulting data, shown in Table III, indicate that the resistance of the water in which the fumigated plants were placed decreased somewhat more rapidly at first than that of the water containing the control plant tissues. This was true for both alfalfa and chard tissues. The fluoride contents of alfalfa roots for the fumigated and control tissues were 15 and 7 ppm, respectively, and the fluoride contents of chard crowns were 25 and 10 ppm, respectively. Data reported earlier indicated that most of the absorbed fluorides are readily extractable by water. It is therefore possible that the differences in resistance were related to the elution of the excess fluoride in the fumigated plants. However, the actual amount of excess fluoride in the fumigated plants over that of controls was just a few micrograms. This amount would not seem sufficient to account for the differences in resistance of the water extracts of the fumigated and control tissues. The plant materials of both species and treatments were carefully rinsed with distilled water before use, so the differences cannot be ascribed to adsorbed conducting materials on the

external surfaces of the tissues. The data therefore suggest that fumigating plants with hydrogen fluoride may increase the permeability of the cell walls to solutes.

Discussion

The studies described here, while significant in themselves, have their greatest value in pointing out where additional studies might yield interesting and important results. The fumigation of plants with hydrogen fluoride during the night indicated that very little fluoride enters the plant if the stomata are closed. The results showed that during night exposure a considerable amount of fluoride is adsorbed on the surfaces of the leaves which can be easily washed off. However, these studies were only carried through five dark periods. Such investigations should be continued for two or three weeks before definite conclusions are drawn concerning intake of fluorides by plants during nighttime. This is important because, in certain cases in the field, atmospheric conditions are such that fumigation of certain areas occur only during the night hours.

The studies on fluoride uptake from the atmosphere by plants whose roots are maintained at different temperatures should certainly be repeated under conditions where the possibility of the contamination of the culture medium can be eliminated. Studies should also be inaugurated in which the uptake of fluorides by plants in different air temperatures can be determined.

Finally, the preliminary results on the effect of absorbed atmospheric fluorides on the permeability of the cell walls indicate that such investigations should be expanded to include many species of plants with a wider range of fluoride contents.

Summary

The results of the three experiments concerning fluoride uptake should be regarded as preliminary but suggestive of additional investigations which should be carried out to verify the results obtained as well as expand the general knowledge of fluoride effects on vegetation. The experiments and the significant results obtained were as follows:

1. Alfalfa plants fumigated with hydrogen fluoride at night accumulated fluoride about as rapidly as plants fumigated during the day for the first three days. However, most of the fluoride accumulated at night could be washed off the plants, indicating it was adsorbed on the surface rather than taken into the plants. After three days, the plants fumigated during the day showed a much more rapid accumulation of fluoride than those fumigated at night.
2. When the roots of plants are maintained in water cultures at 30°C, the uptake of atmospheric fluoride by the tops was much less than when the roots were held at 20°C even though

the air temperature surrounding the tops of the plants was identical. The results suggest that additional studies on temperature effects on fluoride uptake by vegetation would be a fruitful field of investigation.

3. Preliminary results indicate that the cell walls of plant tissues which have absorbed fluoride from the atmosphere have an increased permeability to solutes, based on electrical resistance of water extracts. Again, further study is needed to verify this point.

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Table I--Fluoride Contents of Alfalfa Plants Following Day and Night Hydrogen Fluoride Fumigation (± 0 $\mu g/m^3$)

Total Hours of Fumigation	Time of Fumigation	Fluoride Content of Plants (ppm)			
		Washed			Unwashed
		On Plants*	In Plants	Total	
24	Day	10	24	34	39
24	Night	22	10	32	30
40	Day	21	48	69	78
40	Night	26	16	42	38
LSD**		5	11	12	11

*Amount found in wash water expressed as ppm of the dry weight.

**Least Significant Difference at $P < 0.05$.

Table II--Dry Weight and Fluoride Content of Alfalfa, Orchard Grass, Romaine Lettuce, and Chard, Grown in Constant Air Temperature but in Nutrient Solutions Maintained at 20°C, 25°C, and 30°C, After Two-Month Hydrogen Fluoride Fumigation (0.8 $\mu g/m^3$)

(Each value is the mean of two replications of four plants each)

Plant Species	Roots			Tops		
	20°C	25°C	30°C	20°C	25°C	30°C
			Dry Weight (grams)			
Alfalfa	2.35	0.87	0.89	6.00	3.17	4.00
Orchard grass	0.99	1.05	1.60	3.18	3.95	4.15
Chard	1.07	1.15	0.63	5.85	5.19	4.10
Romaine lettuce	0.38	0.47	0.20	4.10	5.03	6.50
			Fluoride Content (ppm)			
Alfalfa	9	12	27	156	95	59
Orchard grass	69	108	145	172	175	112
Chard	67	80	171	120	90	60
Romaine lettuce	75	83	161	114	97	98

Table III--Change in Resistance With Time of Conductivity Water in Which Identical Quantities of Fumigated and Nonfumigated Plant Tissues Had Been Placed

Time (Minutes)	Electrical Resistance of Solutions (Ohms x 10^5)			
	Alfalfa		Chard	
	Fumigated	Control	Fumigated	Control
0	30.00	30.00	30.00	30.00
5	0.296	0.339	0.334	0.443
10	0.258	0.306	0.294	0.399
15	0.236	0.279	0.268	0.365
20	0.222	0.265	0.248	0.343
30	0.208	0.247	0.227	0.308
40	0.193	0.234	0.211	0.285
50	--*	--	0.199	0.272
60	--	--	0.192	0.256

*Not continued for more than 40 minutes.

A METHOD FOR THE DETECTION AND ISOLATION OF TRACES OF ORGANIC FLUORINE COMPOUNDS IN PLANTS

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INTRODUCTION

During the course of several years of investigating atmospheric fluoride pollution problems we have felt the need for two separate fluoride analytical methods. First, a rapid semiquantitative method for the determination of excess amounts of fluoride in vegetation has been needed; such a method, recently developed in these laboratories, is to be reported elsewhere.[1] Second, a method for the detection and isolation of microgram quantities of organic fluorine compounds from plant materials is the subject of the present paper. The method is presented in two parts: a rapid procedure for screening large numbers of plant extracts and materials for the presence of any organic fluorine compounds, and a chromatographic process for further isolation and identification of materials found in the screening method.

Background

Fluoride analyses of vegetation are confined for the most part to modifications of the Willard and Winter[2,3,4] method. Although this method is reliable and reproducible, it is time consuming, requires relatively large samples (1 to 5 grams vegetation containing at least 5 micrograms of fluoride) and, unless special precautions are taken, is not likely to account for fluorine present in the organic form. At best it gives a total fluoride (organic and inorganic) value. The isolation and identification of fluorine compounds in plant materials to be used as salad crops or

as feed for grazing animals is important because of the known fluoride uptake from polluted atmospheres and because of the very wide range of toxicities, especially of organic fluorine compounds. These toxicities range all the way from inert, innocuous materials (such as the Freons) to poisons (such as fluoroacetic acid and some of the fluoro-olefins where the LD_{50} is in the range that makes detection difficult or impossible). For example, inhalation of the refrigerant Freon CF_2Cl_2 (Freon 12) of 20% concentration does not even produce unconsciousness, although analgesia and confusion result. Recovery is complete within ten minutes.[5] In contrast, the lethal dose of fluoroacetic acid for man is estimated at 2-10 milligrams per kilogram[6,7] body weight. The toxicity of inorganic fluoride is intermediate between these extremes; the toxic dose of sodium fluoride is estimated between 5 and 10 grams, an amount unlikely to be ingested from vegetable contamination. Regular intake of sublethal doses of inorganic fluoride, however, may produce a definite and damaging series of chronic symptoms.[8,9,10,11] It was the concern over these chronic symptoms and the possibility of the presence of even more toxic organic fluorine compounds in fluoridated salad and forage crops that initiated this research.

In attacking these problems, forage plants (alfalfa and orchard grass) and leafy vegetables (chard, spinach, and romaine lettuce) were fumigated with concentrations of about 0.60-0.83 $\mu g/m^3$ of hydrogen fluoride during their entire growth period. These concentrations produced no fluoride-type

markings on the leaves, although a relatively high concentration of fluorides accumulated in them. (30-100 ppm.)

The problems of separating organic from inorganic fluorides were first attacked qualitatively by making several series of extractions, using solvents of widely varying polarity. Regardless of whether the solvents were employed in order of increasing or decreasing polarity, most (over 90%) of the fluoride was found in the most polar solvent (water), indicating the fluoride to be essentially inorganic. However, sufficient fluoride was found in the nonpolar solvents to allow at least the possibility of the presence of organic fluorine compounds.

DISCUSSION

The isolation and identification of organic fluorine compounds from plant or animal materials is complicated by two factors. First, not only is the fluorine present in very small amounts, but it has previously been shown to be present largely in the inorganic form. As a consequence, organic fluorine in fluoride-fumigated plants is present in even smaller amounts, if at all, and hidden by the small but nevertheless swamping amount of inorganic fluoride. Second, organic fluorine compounds do not in themselves constitute a functional class; for example, fluoroacids behave essentially like other acids and fluoroalcohols have the properties of alcohols in general. Consequently, the chemistry of organic fluorine compounds cannot be used in any way to isolate them as a group nor as

tests to indicate their presence. Therefore, every fraction, extract, or compound must be completely freed of inorganic fluoride and then quantitatively analyzed for residual fluorine.

Quantitative analytical methods are all based on the determination of fluoride in ionic form. A large variety of methods for conversion of organic fluoride to analyzable ionic form have been reported. Many conventional methods are summarized by Simons.[12] None of them are applicable in the microgram range and all are too tedious and time consuming for large numbers of samples. In 1955, Schöniger[13] introduced an oxygen flask combustion method for reducing many of the elements in organic compounds to ionic or other analyzable forms. From this rapid and inexpensive method, a very suitable procedure for the determination of fluorine in organic compounds has been developed. It is particularly applicable to the quantitative determination of fluorine in spots developed on paper chromatograms. However, in a search for minute amounts of unknown organic fluorine compounds in the presence of much larger amounts of inorganic fluoride, new procedures were needed.

METHOD

The various solvent and aqueous extracts of fluoridated vegetation were concentrated on a rotovacuum apparatus and first analyzed for total fluoride.

Sub-micro determination of fluorine

A modification of the procedure reported by Soep[14] was used for quantitative sub-micro determination of fluorine. We found, as did Soep, the decoloration of the zirconium - Eriochrome Cyanine R complex to be proportional to fluoride concentration. We also investigated the more sensitive aluminum-morin complex[15] and a number of other metal-dye complex reagents such as sodium 2-(p-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonate-zirconium lake (SPADNS)[16] before standardizing on the Eriochrome complex as most satisfactory for the determination of from 0-3 micrograms of fluoride. A new direct color alizarin complexan method of fluoride analyses reported by Belcher[17,18,19] was not found satisfactory for this work but was used in the semiquantitative procedure to be reported elsewhere.[1]

Apparatus

A 125-ml iodine flask modified with a platinum wire attached to the stopper according to Schöniger[13] plus a microstopcock attached to the bottom of the flask (Fig. 1).

Volumetric flasks, 50 ml size.

Micropipettes, graduated variety 1-5λ, 5-25λ and 20-100λ sizes.

Portable hair dryer for drying spotted strips of paper.

Beckman model DK Ratio Recording Spectrophotometer used at 546 mμ.

Cuvette from Lumetron Photoelectric Colorimeter

The 1-cm cell was small enough to allow adequate filling with

5-ml of solution and was used sideways to give a 32-mm light path. A simple support was provided to hold it in the light path of the Beckman instrument.

Reagents

Solution A: 528.8 mg of Eriochrome Cyanine R dissolved and diluted to 250 ml with water.

Solution B: 76.3 mg of zirconyl chloride octahydrate dissolved in 202 ml of hydrochloric acid (concentrated) and diluted to 500 ml with water.

Reagent: One volume of "A" mixed with two volumes of "B" fresh daily.

Organic fluorine standard: 15.5 mg of sodium difluoroacetate dissolved and diluted to 100 ml with water giving 0.5 μg of fluorine/μℓ of solution.

Laboratory distilled water was upgraded by distilling through an all-quartz, two-stage water purification apparatus.

All materials were stored in polyethylene bottles.

PROCEDURE

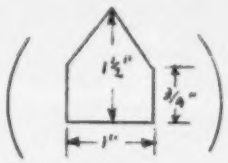
A portion of each extract (about 100 microliters) is spotted on a small piece of filter paper. The paper is dried with hot air from the hair dryer and fixed to the platinum wire of the stopper. After adding

2 milliliters of water, the flask (Fig. 1) is thoroughly flushed with oxygen and the paper strip is ignited from a small pilot flame and plunged into the flask. We found that when the paper is ignited from the top (in the position to be lowered into the flask) a much larger piece containing more sample can be smoothly combusted. When the flame is at the bottom of the strip as it is plunged into the flask it immediately flashes up consuming the whole of the paper and usually causes some of the paper to fall to the water incompletely burned. Also, the more rapid combustion often causes the stopper to be dislodged with resultant loss of gases. The flask is shaken vigorously, drained, shaken with a second 2 milliliters of water, and rinsed with about 0.5 milliliter of water into a 5-milliliter volumetric flask. The freshly mixed reagent is added (0.40 milliliter) and the total volume diluted to 5.0 milliliters. The extinction values are measured at 546 millimicrons after one hour, using water in the reference beam. The values obtained are compared with a calibration curve prepared similarly from the organic fluorine standard (CF_2H-COONa) to obtain the fluorine content. Under these conditions the curve is linear from zero to about two and one-half or three microns of fluoride.

Separation of organic fluoride

Rapid screening procedure

If the above total fluoride analysis (organic or inorganic) shows fluorine to be present, a separation must be accomplished to determine if any of it is in the organic form. The method used for this separation

separation was developed from chromatographic procedures for separation of inorganic anions and from the elution concentration scheme of Davis, Dubbs and Adams[20] and was designed to screen large numbers of plant extracts. First, a small section was cut from a 1-inch roll of No. 1 Whatman chromatographic paper 

and a small volume of extract (volume dependent on previous total F analysis) spotted along the base of the wedge. The sample can be placed either in a spot or along a line across the base. We did not find it necessary to elute contaminants on the paper and to trim off the tip before using, as did Davis and coworkers, because, although materials were found there, they did not interfere with the subsequent fluoride analysis. We found it convenient to use full length microscope slides rather than cut them in half as did Davis and coworkers. After clamping with No. 2 ring clips, obtainable in any stationery store, ten units can be placed in a standard staining jar without support (Fig. 2). A chromatographic solvent or solvent mixture in which fluoride ion has been determined not to move ($R_F = 0$) is placed in the jar. The solvent immediately fills the space between the two slides, providing a continuous source to the base of the strip. The edges of the dish are covered to reduce evaporation from other than the tips, and the strips allowed to stand overnight. The solvent evaporates from the tip allowing a continual flow of fresh solvent and thereby causing even the slow moving (R_F low) substances to move to the tip and be concentrated there while inorganic fluoride remains on

the original spot. Several solvent mixtures for the separation of the halides and other anions are listed in McOmie and Pollard.[21] We used as standard the upper layer of a mixture of equal parts of n-butanol and 1.5N ammonia, well shaken and allowed to stand at least overnight before separation. This solvent mixture was exhaustively tested to be sure that the R_F factor for inorganic fluoride was consistently zero. In addition, papers spotted with sodium fluoride were run alongside every batch of plant extracts and the fluoride was found in all cases to remain on the original spot. After overnight development, a small portion of the tips and the original spot are separately combusted in the oxygen flask and analyzed for fluoride as above. Under these conditions, inorganic fluoride remains exactly on the original spot and all of the organic fluorine compounds tested move quickly to the tip (Table I). Any fluoride found in the combustion of the tips (with butanol solvent) is therefore definite indication of the presence of a fluoro-organic. As confirming evidence of organic fluorides, duplicate strips were run using water as solvent rather than butanol-ammonia. Here, of course, inorganic fluoride quickly moves to the tip. The movement of fluoro-organics with water is not so straightforward and depends on the nature of the compounds. Highly polar, water-soluble compounds would move with the solvent and nonpolar insoluble materials would remain in the original spot. The butanol and water strips together give an indication of the presence and nature of an organic fluorine compound. Table I indicates the results of organic and inorganic fluorides run on short-strip analysis.

Profile analyses of chromatograms for organic fluorine compounds

It should be obvious, of course, in the short-strip analysis with butanol-ammonia, that essentially all of the organic compounds present move to the tip and not merely the fluoro-organics. Thus, an absolute separation of inorganic from organic fluorine compounds has been obtained, but no indication of the number or nature of the organic compounds present.

If a positive indication of fluoro-organics is obtained in the short-strip analysis, a number of conventional paper chromatographic strips are run. For example, a portion of an extract showing an indication of an organofluorine compound in the short-strip analysis is spotted on a 1" x 18" strip of No. 1 Whatman chromatographic paper. The spot is dried and the strip hung in a chromatographic tank overnight in equilibration with the vapors of the lower butanol-1.5N ammonia layer mentioned above. The strip is then developed several hours with the upper layer of the same solvent mixture and thoroughly dried. Theoretically, the strip could be treated with a suitable reagent to make visible the organic fluoride sought. Unfortunately several factors preclude this: first, as mentioned above, there are no color reactions for the general fluoro-organic class; second, the compounds, if any, are present in extremely small amounts; third, the paper chromatogram is rather completely stained yellow and green with chlorophyll and other pigments.

Instead, the chromatogram is subjected to a procedure we have chosen to call a profile analysis. It must be quantitatively analyzed for fluorine by sections. A short chromatogram of 12 to 15 centimeters is cut crosswise in ten strips, each representing 0.1 R_F factor. A larger strip might be cut in twenty strips of R_F 0.05 each. Each strip is combusted in the oxygen flask and analyzed for fluoride. A profile of the fluoride content of the strip is then obtained by plotting the fluoride analyses vs. the R_F factor (Fig. 3). In the chromatogram represented in Fig. 3a a sample of sodium difluoroacetate containing 10 μg of fluorine was spotted and developed as described. The dried paper was analyzed in twenty strips, each representing 0.05 R_F units. As indicated in the figure, the R_F factor for $CF_2HCOONa$ is about 0.25. The strips at R_F 0.25, 0.30 and 0.35 indicate a total of about 7 mg of fluorine, or 70% recovery. In the chromatograph represented in Fig. 3b, a mixture of sodium fluoride and sodium difluoroacetate were spotted together and developed, again in butanol-ammonia, but a little longer for better separation. The inorganic fluoride is found in the R_F 0.05 and 0.10 strips and the organic fluorine again found at R_F 0.20 to 0.25. The strip at R_F 0.15 is free of fluorine, indicating complete separation of the two compounds. Fig. 3c shows the profile analysis of a solvent extract of hydrogen fluoride fumigated orchard grass indicating the presence of inorganic fluoride and possible traces of two organic fluorine compounds. Since the strips are cut from the chromatograph by R_F number and not by visual location of any spot, a single compound will usually be found in two or three adjacent strips rather than a single one.

The final step is to isolate the material in pure form. An obvious method is to prepare a number of chromatograms under identical conditions, determine the location of the unknown on one chromatogram by profile analysis and extract the material from similar R_F portions of the remainder. This method was applied as follows. A portion of fumigated orchard grass tips (fluoride previously shown to concentrate there) was extracted in a soxhlet with petroleum ether followed by ether and several other solvents. The ether extract was found to contain 1.2 micrograms total fluoride per 100 microliters. A short-strip analysis indicated the fluoride to be approximately 70 percent organic. Therefore, a portion of the ether extract was spotted on a 1" x 18" strip of paper, equilibrated, and developed with butanol-ammonia. Combustion of the resulting chromatogram in twenty strips (0.05 R_F units) gave a peak at R_F 0.0-0.1 for inorganic fluoride and a larger peak at R_F 0.7-0.9 indicating organic fluoride. To confirm the finding of organic fluoride at R_F 0.7-0.9, eighteen strips $3\frac{1}{4}$" wide were each spotted with 500 gamma of the extract and developed in butanol-ammonia as before. The 0.7-0.9 portion of each strip was cut out, taking care to eliminate most of the chlorophyl portions which run with solvent front. These portions were extracted with ether in a soxhlet and the extract concentrated to about 2 milliliters. This extract was found to contain 6-8 micrograms total fluoride per 100 microliters. Short-strip analysis indicated it all to be organic.

Larger quantities of these fluoridated salad and forage crops were investigated by chromatography using heavier preparative papers and cellulose column techniques. Although microgram quantities of organic fluorine compounds were found, the levels were far too low for isolation and identification or to cause concern over toxic materials.

ACKNOWLEDGEMENTS

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SUMMARY

A method for the detection and isolation of submicrogram quantities of organic fluorine compounds from plant materials in the presence of much larger amounts of inorganic fluoride is presented. The procedure consists first of a rapid screening step for use with large numbers of vegetable samples and extracts and, second, of a chromatographic step to isolate and characterize any fluoro-organics found. These methods are developed in light of specific chemical characteristics of organic

fluorine compounds as a general class. A modification of Soep's quantitative sub-micro fluoride analytical method is presented as applicable to these isolation methods. Microgram quantities of organic fluorine compounds were found in the plant materials investigated but at a level too low for isolation and identification.

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Table I

SHORT-STRIP FLUORIDE ANALYSIS

Compound or Extract	Solvent	Micrograms of Fluoride	
		Tip	Spot
Perfluorobutyric acid	Butanol	3.2	0.3
Perfluorobutyric acid	H_2O	3.2	0.2
Sodium fluoroacetate	Butanol	3.0	0
Sodium fluoroacetate	H_2O	3.5	0.1
Sodium difluoroacetate	Butanol	0.8	0.2
Sodium fluoride	Butanol	0	2.9
Sodium fluoride	H_2O	3.6	0.1
2,4-Dinitrofluorobenzene	Butanol	0.6	0.3
2,4-Dinitrofluorobenzene	H_2O	0.9	0

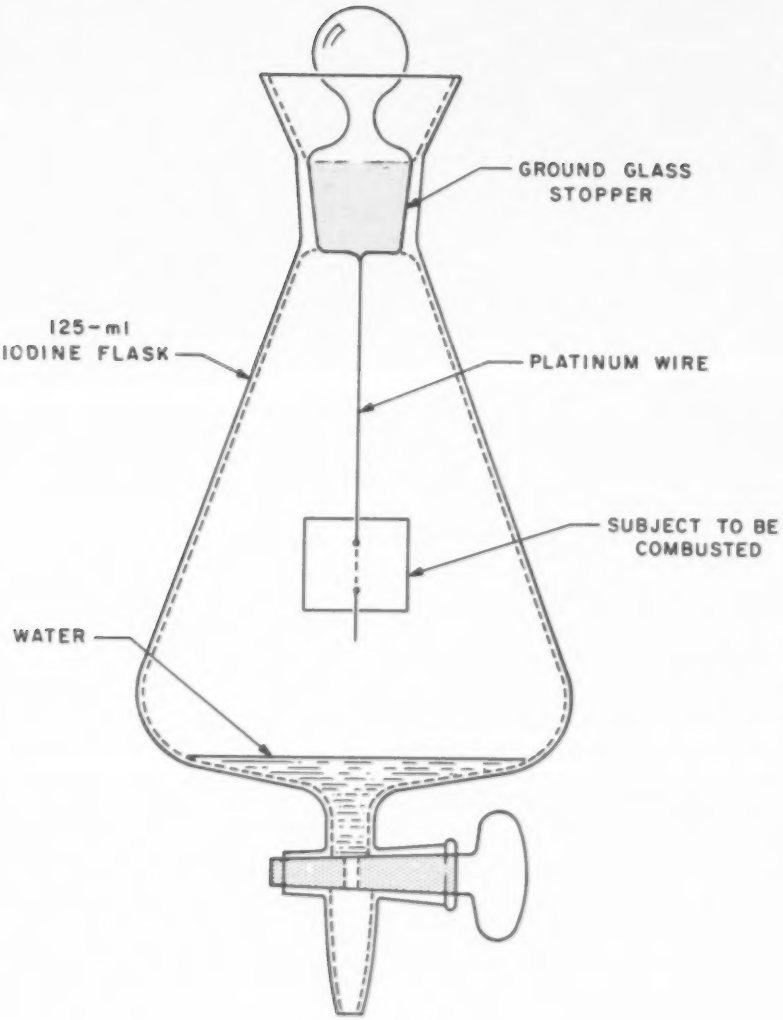

Fig. 1 Combustion apparatus

Fig. 2 Short-strip separations of organic from inorganic fluoride

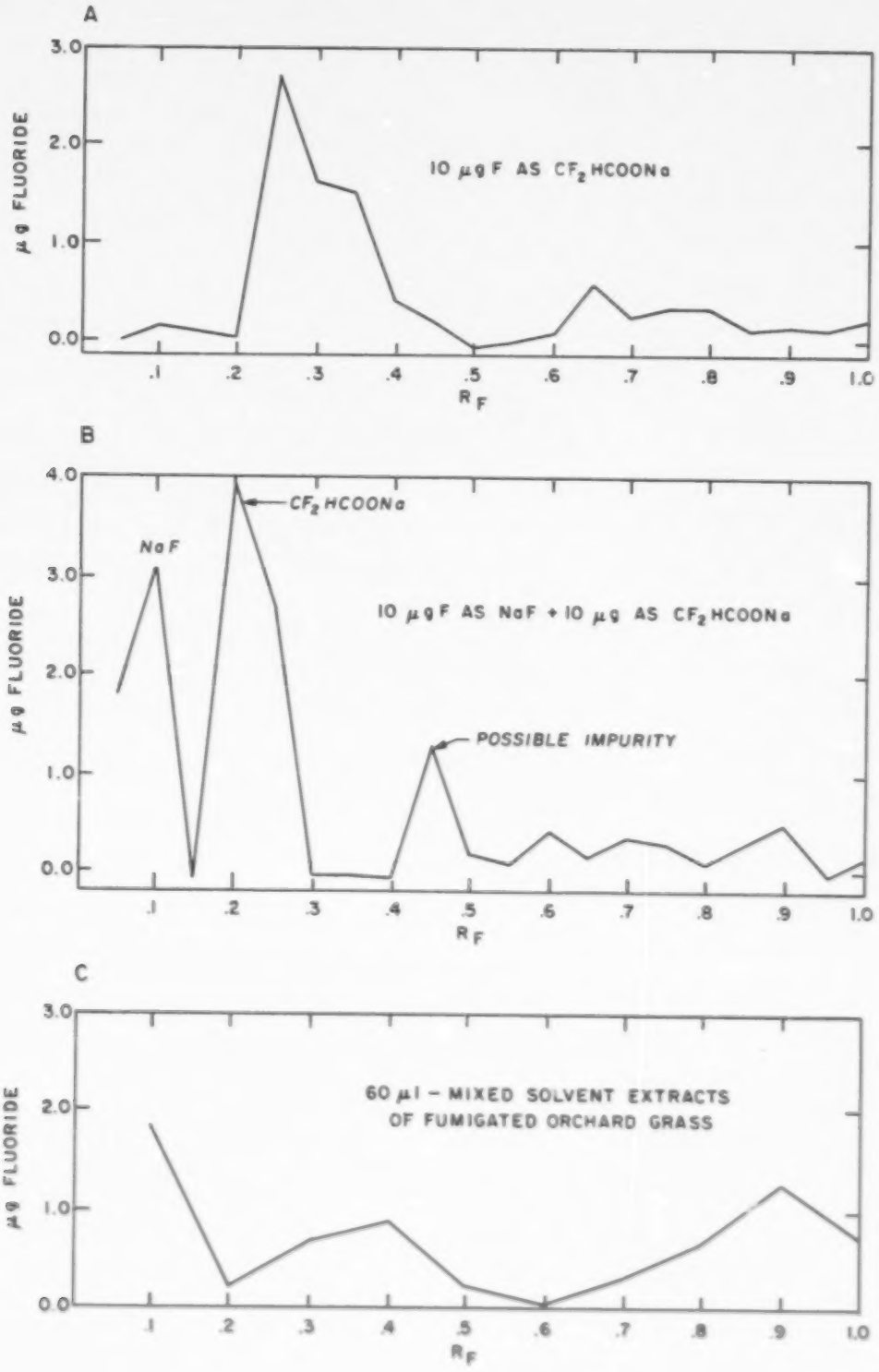

Fig. 3 Profile analysis of fluoride in chromatographic strips

A RAPID FIELD METHOD FOR DETERMINATION OF FLUORIDE IN PLANTS*

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During the course of several years investigation of fluoride pollution problems, we have felt the need for methods that were much less precise--even qualitative rather than quantitative in nature--and that would permit a rapid determination as to the presence or absence of excess amounts of fluorine-bearing compounds. Thus, in walking through an orchard, one might notice that leaves of apricots or Italian prunes have what we cautiously call fluoride-type markings. If a spot test were available whereby an investigator could readily demonstrate a higher than normal level of fluoride in the leaves, his ability to correctly diagnose the symptoms would be greatly enhanced.

In reviewing the methods for fluoride detection, it was found that a colorimetric reaction into which the fluoride ion enters directly, producing a new color, has been reported by Belcher, Leonard, and West (1). Previously available tests involved the bleaching effect of fluoride on

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a highly colored metal complex or the formation of a colorless metal-fluoride complex with the liberation of free dye. Belcher and West (2) have studied extensively this new reaction which involves the cerium^{III} complex of alizarin complexan, 3 di(carboxymethyl)aminomethyl -1,2-dihydroxyanthraquinone with respect to optimum pH, time of stable color development, and the effect of interfering ions. A test has been described by Belcher et al. (3) for the detection of fluorides using the cerium^{III}-alizarin complexan reagent.

Herein described is a simple field method for the semiquantitative detection of microgram quantities of fluorides in plant tissues without the need for preliminary drying, fusion and/or combustion, or distillation.

REAGENTS

(a) Alizarin complexan* solution.--192.5 milligrams suspended in water and dissolved with a minimum of 10% NaOH, brought to pH ~ 5 (red color) with dilute HCl and diluted to 500 milliliters.

(b) Cerous nitrate.--0.002M.

(c) pH 4.0-4.5 buffer.--7.5 grams NaOAc and 7.5 milliliters HOAc in 1 liter of water.

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* The reagent can be obtained from Hopkin and Williams Ltd. Essex, England.

DETERMINATION

A small amount of fresh plant material (~2 grams) is macerated in a Pyrex mortar and pestle with the aid of some finely ground, acid-washed silica sand and ~4 milliliters distilled water. The liquid phase of the resulting aqueous suspension is filtered through paper pulp to produce a clear extract. One or two drops of this extract are placed directly in the depression of a spot plate. To this extract are added, in succession, one drop of sodium acetate-acetic acid buffer, one drop of 0.001M alizarin complexan solution and, with immediate stirring, one drop of 0.002M cerous nitrate. The mixture should be allowed to stand for 10 minutes out of direct light. A comparison of the test sample with the red color of a blank, using only the reagents and water, should reveal a definite blue color if fluoride is present in a concentration of 40 parts per million or greater. The deeper the blue, the greater the concentration up to a limiting value of ~200 ppm.

To accomplish the filtration of plant extract in the field, a simple filtering tube may be prepared from lab glassware. A number of these tubes may be prepared at one time for use outside the lab. A small hole is first made in the bottom of a large test tube (25 x 200 mm). Into the tube is poured a slurry of paper pulp such that the final volume will fill about 3/8" of the bottom of the tube. A glass rod flattened at one end is used to press the paper pulp to the bottom and the pulp sucked dry with a vacuum pump. In order to effect the filtration, a plunger is made from a rubber

stopper and a length of glass tubing. The rubber stopper is cut so that the large end fits tightly into the tube. A groove is cut around the stopper near the smaller end so that it may be fitted to the end of the glass tubing which has been heated and closed slightly. The macerated plant sample is poured directly over the paper pulp and, after wetting the rubber plunger, enough air pressure can be created inside the tube to force the liquid portion through the paper pulp.

The cerium^{III}-alizarin complexan reagent is sensitive to 0.5 microgram fluoride ion in 0.005 milliliter, but the color reaction is inhibited by a number of interfering ions which are present in the plant extract. Certain anions react with the reagent by removing the cerium^{III} from the alizarin complexan. This was overcome by using sufficient cerous nitrate to provide an excess of free cerium ions. Heavy metals also cause interference when present in amounts greater than the fluoride concentration. This is the main limiting factor in the sensitivity of the reaction using plant tissue extracts. Ion exchange resins have been effectively used in the laboratory to eliminate these interferences and increase the sensitivity, but their use complicates the simplicity of a field technique.

Sensitivity is also effected by the pH of the mixture. Optimum sensitivity occurs at pH 5.0-5.2(2); however, for the purpose of this test, pH 4.0-4.5 gives a stronger and more consistent result.

Orchard grass (Dactylis glomerata) was used almost exclusively for the study of this method because fluoride accumulates in the tips of the

blades and a small sample size is all that is required to positively determine if fluoride is present. A survey of other commonly occurring plant materials gave equally satisfying results.

SUMMARY

A colorimetric reaction, involving the cerium^{III}-alizarin complexan reagent, is described for the simple semiquantitative determination of excess amounts of fluoride. A method is outlined that may be used in the field for the determination of fluoride in plants in excess of 40 ppm.

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